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Mercury inhibits the non-photochemical reduction of plastoquinone by exogenous NADPH and NADH: evidence from measurements of the polyphasic chlorophyll *a* fluorescence rise in spinach chloroplasts

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Abstract

Chlorophyll *a* fluorescence rise kinetics (from 50 μs to 1 s) were used to investigate the non-photochemical reduction of the plastoquinone (PQ) pool in osmotically broken spinach chloroplasts (*Spinacia oleracea* L.). Incubation of the chloroplasts in the presence of exogenous NADPH or NADH resulted in significant changes in the shape of the fluorescence transient reflecting an NAD(P)H-dependent accumulation of reduced PQ in the dark, with an extent depending on the concentration of NAD(P)H and the availability of oxygen; the dark reduction of the PQ pool was saturated at lower NAD(P)H concentrations and reached a higher level when the incubation took place under anaerobic conditions than when it occurred under aerobic conditions. Under both conditions NADPH was more effective than NADH in reducing PQ, however only at sub-saturating concentrations. Neither antimycin A nor rotenone were found to alter the effect of NAD(P)H. The addition of mercury chloride to the chloroplast suspension decreased the NAD(P)H-dependent dark reduction of the PQ pool, with the full inhibition requiring higher mercury concentrations under anaerobic than under aerobic conditions. This is the first time that this inhibitory role of mercury is reported for higher plants. The results demonstrate that in the dark the redox state of the PQ pool is regulated by the reduction of PQ via a mercury-sensitive NAD(P)H-PQ oxidoreductase and the reoxidation of reduced PQ by an O_2-dependent pathway, thus providing additional evidence for the existence of a chlororespiratory electron transport chain in higher plant chloroplasts.

Abbreviations: Chl *a* – chlorophyll *a*; F_M – maximal fluorescence yield; F_0 – minimal fluorescence yield; F_V – maximal variable fluorescence yield, equal to $F_M - F_0$; FNR – ferredoxin-$NADP^+$ oxidoreductase; FQR – ferredoxin-PQ oxidoreductase; I and J – intermediate steps in the Chl *a* fluorescence transient appearing between F_0 and F_M; PS I – Photosystem I; PS II – Photosystem II; PQ – plastoquinone; Q_A – primary quinone electron acceptor of PS II; RC – reaction centre

Introduction

The reduction of the plastoquinone (PQ) pool in higher plant chloroplasts is a well documented light-driven process. Normally, the PQ molecules that become reduced by the PS II activity during illumination are reoxidised at a high rate when the sample is transferred to darkness. On the other hand, there are reports showing that PQ accumulates in its reduced form following a light-to-dark transition (Asada et al. 1993; Groom et al. 1993; Feild et al. 1998) and when leaves are exposed to anaerobic conditions in the dark (Harris and Heber 1993; Farineau 1999; Haldimann and

Strasser 1999). Non-photochemical PQ reduction and regulation of the redox state of the PQ pool by O_2 were observed in algae long ago (Goedheer 1963; Schreiber and Vidaver 1974). However, it was only after the work of Bennoun (1982), who introduced the term chlororespiration and proposed a model for a hitherto uncharacterised respiratory electron transport chain in the thylakoid membranes of green algae, that numerous studies were devoted to this subject (for reviews see Scherer 1990; Büchel and Garab 1997). According to the model of Bennoun (1982) chlororespiration is defined as consisting of the non-photochemical reduction of the PQ pool by an NAD(P)H-PQ oxidoreductase and a subsequent dark reoxidation of reduced PQ by a putative terminal oxidase leading ultimately to the reduction of molecular oxygen. However, the existence of a PQ-oxidase in the thylakoid membranes is only supported by indirect evidence provided by biophysical measurements, while molecular proof remains to be provided (see Scherer 1990; Garab et al. 1989; Büchel and Garab 1997; Feild et al. 1998). Moreover, there is still a debate concerning the non-photochemical reduction of the PQ pool, with reports supporting the suggestion that it is mediated by an NAD(P)H-PQ oxidoreductase and others that challenge it and propose other pathway(s). The discovery that the chloroplast genome of several plant species comprises *ndh* genes, the sequences of which show homologies to genes encoding subunits of the mitochondrial NADH-ubiquinone oxidoreductase or complex I (Shinozaki et al. 1986; Sugiura 1992), provided a first indication that an NAD(P)H-PQ oxidoreductase is involved in the dark reduction of PQ. Recently, a functional NADH-dehydrogenase (Ndh complex), encoded by the plastid *ndh* genes and showing analogies to complex I, has been isolated from pea (*Pisum sativum*) thylakoid membranes (Sazanov et al. 1998b). Furthermore, studies with mutants containing disrupted *ndh* genes have demonstrated that the plastid Ndh complex is involved in the post-illumination reduction of the PQ pool (Burrows et al. 1998; Endo et al. 1998; Kofer et al. 1998; Sazanov et al. 1998a; Shikanai et al. 1998). On the other hand, there are several reports postulating that only NADPH (and not NADH) can reduce the PQ pool in the dark and that the reduction is predominantly, if not exclusively, realised via an electron transport pathway involving ferredoxin-$NADP^+$ oxidoreductase (FNR) and a hitherto uncharacterised ferredoxin-PQ oxidoreductase (FQR) (Bendall and Manasse 1995; Endo et al. 1997, 1998). It has also been reported that this NADPH-specific non-photochemical reduction of PQ is sensitive to antimycin A (Mills et al. 1979; Endo et al. 1997, 1998).

It is generally accepted that chlorophyll (Chl) *a* fluorescence rise kinetics reflects the closure of the reaction centres (RCs) of Photosystem II (PS II), i.e. the reduction of Q_A to Q_A^-, and can therefore provide information on the photochemical activity of PS II and the associated filling up of the PQ pool (Krause and Weiss 1991; Dau 1994; Govindjee 1995). When dark-adapted leaves are illuminated at room temperature with a high-intensity actinic light (about 600 W m^{-2}), the Chl *a* fluorescence rise they exhibit, accomplished in less than 1 s, is polyphasic (Neubauer and Schreiber 1987; Schreiber and Neubauer 1987; Strasser et al. 1995) with two intermediate steps between the minimum yield F_0 and the maximum yield F_M. The two steps, more clearly revealed when the fluorescence kinetics is plotted on a logarithmic time scale (Strasser and Govindjee 1992; Strasser et al. 1995), appear at about 2 and 30 ms and are called, respectively, I_1 and I_2 (Neubauer and Schreiber 1987) or J and I, hence the notation OJIP for the fluorescence transient (Strasser and Govindjee 1992; Strasser et al. 1995).

As the OJIP transients reflect, with a high time-resolution, the dynamic variation of the concentrations of the various redox states of the PS II units, they were recently employed in order to probe the non-photochemical PQ reduction in pea leaves (Haldimann and Strasser 1999). The changes of the shape of the fluorescence transient observed after the leaves were exposed to anaerobic conditions in the dark, and especially the dramatic increase of the fluorescence signal in the region of the J-level, reveal an accumulation of reduced PQ. Such changes could also be induced by anaerobiosis in osmotically broken pea chloroplasts, but only when exogenous NADPH or NADH was added to the chloroplast suspension, while neither NADPH nor NADH had any effect on the shape of the fluorescence transient OJIP under aerobic conditions (Haldimann and Strasser 1999). These latter findings differ from those obtained in studies conducted with broken spinach (*Spinacia oleracea*) chloroplasts where NADPH specific non-photochemical PQ reduction was observed under aerobic conditions (Endo et al. 1997).

In the present work, we applied the experimental approach employed for the case of pea chloroplasts (Haldimann and Strasser 1999) to spinach chloroplasts and we indeed verified that they behave differently. It will be shown that in osmotically broken spinach

chloroplasts PQ is reduced even under aerobic conditions in the dark, however by both forms of reduced pyridine nucleotides and not specifically by NADPH. It will be further shown that anaerobiosis strongly enhances this NAD(P)H-dependent non-photochemical accumulation of reduced PQ. An important finding is that the NAD(P)H-dependent dark reduction of the PQ pool in broken spinach chloroplasts is strongly inhibited by mercury, both under aerobic and anaerobic conditions. This is the first time that mercury is found to suppress the dark reduction of PQ in higher plant cells. Our results provide additional evidence for the existence of a chlororespiratory electron transport chain in higher plant chloroplasts.

Materials and methods

Chloroplast isolation

Chloroplasts were isolated at 4 °C from spinach (*Spinacia oleracea* L.) purchased from local markets. Sliced leaves were homogenised for 10 s in a Waring Blender in a grinding medium containing 330 mM sorbitol, 50 mM MES-KOH (pH 6.1), 30 mM KCl, 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$ and 0.5 mM KH_2PO_4. The slurry was filtered through two layers of Nylon (36 μM) plus two layers of muslin cloth. The pellet obtained after centrifugation at 2000 $\times$ *g* for 5 min and 4 °C was suspended in 2 ml of the same medium and layered on 40% Percoll (v/v) in the same medium. The pellet with intact chloroplasts, obtained after centrifugation of the Percoll-containing tubes at 4000 $\times$ *g* for 10 min and 4 °C, was resuspended in a medium containing 330 mM sorbitol, 5 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA and 50 mM HEPES-KOH (pH 7.5) and centrifuged to remove the Percoll. The pellet was resuspended in 1–2 ml of the same medium. The chloroplasts were osmotically ruptured by resuspension in 30 mM $MgCl_2$ for 5 min followed by the addition of an equal volume of double strength suspension medium. Measurements were made at a chlorophyll (Chl) concentration of 50 μg Chl ml^{-1}. The Chl concentration was measured according to the method of Arnon (1949).

Treatment of the chloroplasts with NAD(P)H

For the treatment with reduced pyridine nucleotides, different concentrations of NADPH or NADH were added to 500 μl aliquots of chloroplast suspension (50 μg Chl ml^{-1}) in 1 cm diameter vials with a total volume of 2 cm^3. Thereafter, the samples were incubated for 60 min at room temperature in the dark either under aerobic or anaerobic conditions. Anaerobic conditions were induced by flushing the samples with N_2 gas and adding 10 mM glucose, 50 units ml^{-1} glucose-oxidase and 1000 units ml^{-1} catalase to the chloroplast suspension. The vials were then immediately sealed with air tight caps. NADPH and NADH were purchased from Sigma or from Boehringer Mannheim.

The choice of the 60-min incubation time with the reduced pyridine nucleotides (see also Haldimann and Strasser 1999), both in the absence and presence of any of the tested inhibitors, was based on preliminary experiments which showed that, though similar results were obtained from measurements after a 15-min incubation as after 60 min, prolonged incubation time improved reproducibility of the results among replicates.

Chlorophyll a *fluorescence measurements*

Chlorophyll *a* fluorescence transients were recorded at room temperature using a PEA-fluorometer (PEA, Hansatech, King's Lynn, Norfolk, UK) as described elsewhere (Strasser et al. 1995; Srivastava et al. 1995). The measurements were performed on 500 μl aliquots of chloroplast suspension (50 μg Chl ml^{-1}) in 1 cm diameter vials as described elsewhere (Haldimann and Strasser 1999). The excitation light of an intensity of 600 W m^{-2} (about 3200 μE m^{-2} s^{-1}) was provided by an array of six light-emitting diodes (red light, peak at 650 nm). The Chl *a* fluorescence signal was detected using a PIN photocell after passing through a long pass filter (50% transmission at 720 nm). The Chl *a* fluorescence signals were recorded in a time span from 10 μs to 1 s at a 12 bit resolution with a data acquisition rate of 10 μs for the first 2 ms and 1 ms between 2 ms and 1 s. The fluorescence signal at 50 μs, being the earliest measurement free of artefacts related to the electronics of the instrument, was considered as F_0. The fluorescence transients were plotted on a logarithmic time scale.

Results

Effects of NADPH and NADH on the kinetics of the polyphasic Chl a *fluorescence rise*

Figure 1 presents a set of Chl *a* fluorescence rise kinetics (from 50 μs to 1 s) exhibited by osmotically broken

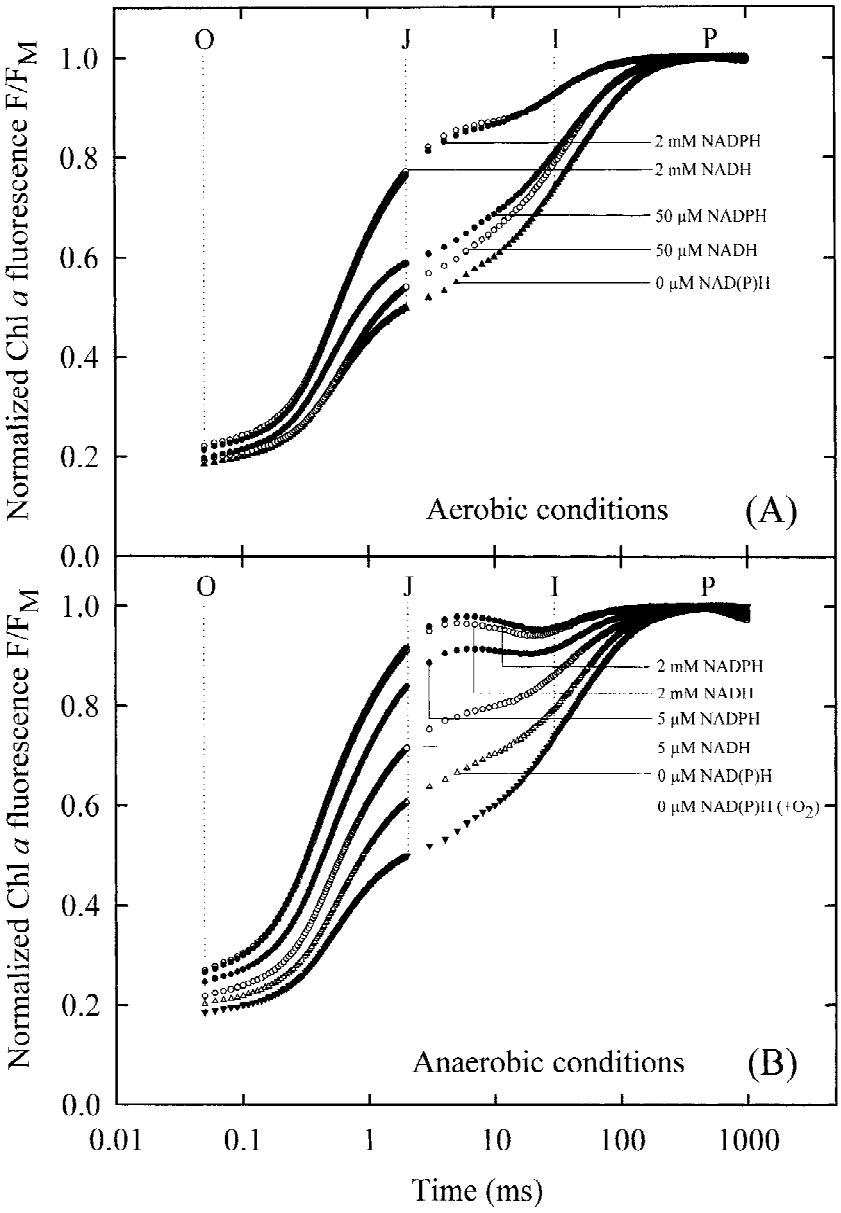

Figure 1. Chl *a* fluorescence rise kinetics of osmotically broken spinach chloroplasts incubated for 60 min at room temperature in the dark with or without the addition of different concentrations of NADPH or NADH to the suspension medium. The incubation took place both under aerobic (A) and anaerobic conditions (B). In (B) the first curve from the bottom was obtained from a sample incubated under aerobic conditions (+ O_2) without the addition of NAD(P)H to the suspension medium. The chlorophyll concentration was 50 μg Chl ml^{-1}. The transients are normalised on the corresponding F_M.

spinach chloroplasts that were incubated at room temperature in the dark in a medium comprising reduced pyridine nucleotides (NADH or NADPH) of different concentrations, both under aerobic (Figure 1A) and anaerobic conditions (Figure 1B). The transients of the corresponding control samples, i.e. the samples where no NAD(P)H was added, are also depicted. All transients are plotted on a logarithmic time scale, so that their polyphasic shape could be clearly revealed (Strasser and Govindjee 1992; Strasser et al. 1995). In intact leaves, intact chloroplasts and algae the fluorescence rise exhibits two intermediate steps between F_0 (O) and F_M, at about 2 and 30 ms, denoted as J (F_J) and I (F_I), respectively (Strasser et

al. 1995). However, as clearly shown in Figure 1 and also observed in a previous study conducted with pea (Haldimann and Strasser 1999), the transients of the osmotically ruptured chloroplasts do not exhibit the second intermediate step, i.e. the I-step. Nevertheless, the fluorescence signals at 30 ms (F_I) were retained as characteristic of the J–P phase of the transients. The fluorescence signals in Figure 1 were normalised on the corresponding F_M in order to facilitate the comparison of the transients. This normalisation can be well considered as reasonable, since the absolute values of the maximal fluorescence yield F_M were found to differ by less than 10% among all samples tested (data not shown).

As shown in Figure 1A which refers to the aerobic conditions, in the presence of any of the two forms of reduced pyridine nucleotides all the fluorescence values of the normalised transient leading to the maximum P (=1) exhibit an increase, which is however much more pronounced in the region of the J-step, indicating an increase in the fraction of reduced PQ (Haldimann and Strasser 1999). The amplitude of the changes was found to depend on the concentration of NAD(P)H, as shown for two concentrations of each pyridine nucleotide in Figure 1A. Data for the full range of NAD(P)H concentrations will be presented later in Figure 2. Figure 1A also demonstrates that the addition of NAD(P)H at near-saturating concentrations resulted in a delay in the appearance of the first intermediate step (around 5 ms instead of 2 ms). However, we used fluorescence values at 2 ms for consistency in recording intensity at a fixed time.

It has to be pointed out that, though the results concerning the amplitude of the changes are highly reproducible in repetitive measurements conducted on the same chloroplast preparation, variations of the amplitude were observed among preparations conducted on different days. For example, one can see that the changes induced by 2 mM NADPH in one chloroplast preparation (Figure 1A) are less than those caused by 500 μM NADPH in another preparation (shown later in Figure 3A). A future investigation is needed to explain the origin of this finding which, for the moment, we speculate that it might be due to possible losses of biochemical components, with an extent depending on the physiological condition of the leaves. Despite the differences between preparations with respect to the amplitude of the NAD(P)H induced changes, the trend of the changes, i.e. an increase of their amplitude with increasing NAD(P)H concentration, was always observed. It is also of note that in some preparations the increase of the fluorescence yield in the I–P phase of the transient in the absence of NAD(P)H was slower in the sample incubated under anaerobic conditions than in that kept under aerobic conditions (cf. Figure 1 with Figure 3, shown later). For the moment we have no clue to explain this observation or why no such difference appeared in other preparations (Figure 1).

Under anaerobic conditions the characteristics of the fluorescence transient were modified, with the changes resembling those induced by moderate concentrations of NAD(P)H. This is clearly shown in Figure 1B which, though referring to anaerobic conditions, includes also the transient of the control of the aerobic conditions (0 μM NAD(P)H +O_2) to facilitate the comparison with the transient of the control of the anaerobic conditions (0 μM NAD(P)H).

Furthermore, anaerobic conditions strongly enhanced the NAD(P)H-induced modifications of the fluorescence rise transient as demonstrated by the comparison of Figures 1B and 1A, reflecting a further accumulation of reduced PQ. The pronounced increase of the fluorescence signal at the J-level is followed by a clear dip in the J-P phase, in the region of F_I (Figure 1B).

The enhancement of the NAD(P)H induced changes by anaerobic conditions is even more prominent at low NAD(P)H concentrations. Under anaerobic conditions large changes of the kinetics of the Chl *a* fluorescence rise appeared already at an NAD(P)H concentration of 5 μM, whereas under aerobic conditions a ten times higher concentration resulted in less pronounced changes. Under both aerobic and anaerobic conditions, NADPH was more effective than NADH in altering the shape of the fluorescence curve, although only at the lower concentration range.

The NAD(P)H-dependent dark reduction of PQ was accompanied by an increase in the minimal fluorescence yield F_0. This can be due to an incomplete re-oxidation of Q_A^- in the dark due to equilibration with the highly reduced PQ pool. Another possible explanation is that the proposed quenching effect of oxidised PQ on F_0 (Vernotte et al. 1979) is here eliminated due to the reduction of a big fraction of PQ in the dark. However, the extent of F_0 increase was found to be rather limited under anaerobic conditions (Figure 1B) and even smaller under aerobic conditions (Figure 1A). This means that even if the redox state of PQ affects the redox state of Q_A in the dark, Q_A remains largely oxidised even when a large fraction of PQ is reduced.

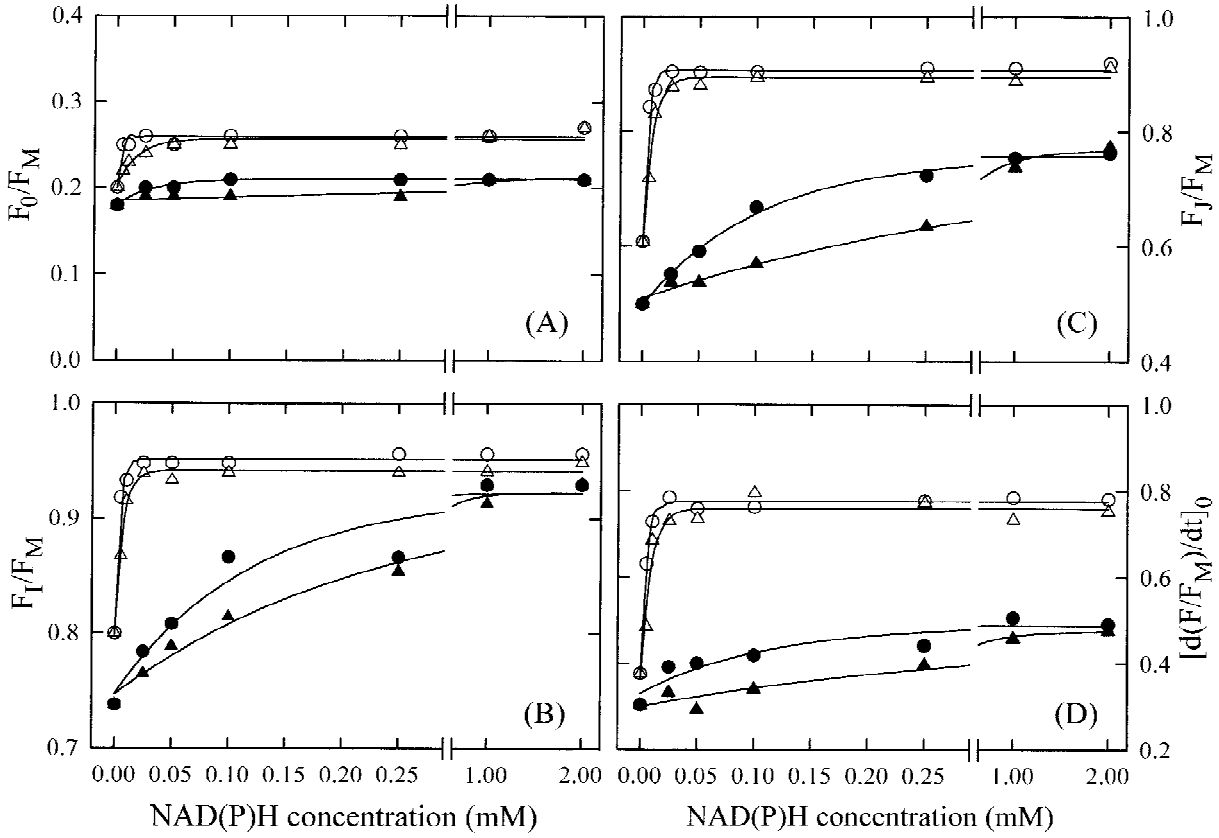

Figure 2. Effects of different concentrations of NADPH (●, ○) or NADH (▲, △) on the following parameters, characteristic of the Chl *a* fluorescence transient, all referring to transients normalised, as in Figure 1, on the corresponding F_M: (A) F_0/F_M, the minimal fluorescence yield, at 50 μs; (B) F_I/F_M, the fluorescence yield at the I-step (30 ms); (C) F_J/F_M, the fluorescence yield at the J-step (2 ms); (D) $[d(F/F_M)/dt]_0$, the initial slope of the normalised fluorescence rise, approximated by $[\Delta(F/F_M)/\Delta t]$ between 50 and 300 μs. The experiment was conducted both under aerobic (●, ▲) and anaerobic (○, △) conditions. The selected parameters were calculated from the fluorescence transients obtained from osmotically broken spinach chloroplasts that were incubated for 60 min at room temperature in the dark. The chlorophyll concentration was 50 μg Chl ml^{-1}.

In order to visualise in a quantitative way the shape changes induced by the different concentration of NAD(P)H, we followed characteristic parameters of the normalised Chl *a* fluorescence transients. The normalisation was done over F_M, the only fluorescence signal that appeared insensitive to the different treatments. The chosen parameters are the fluorescence values at the steps O (initial), J (at 2 ms) and I (at 30 ms), i.e. F_0/F_M, F_J/F_M and F_I/F_M respectively, as well as the initial slope $[d(F/F_M)/dt]_0$ of the fluorescence rise, approximated by $[\Delta(F/F_M)/\Delta t]$ between 50 and 300 μs. The latter provides an estimate of the initial rate of RC closure i.e. of the accumulation of Q_A in its reduced form Q_A^-, which is the net result of Q_A reduction by the PS II activity and its reoxidation by the oxidised PQ. The values of the four parameters are plotted in Figure 2 *versus* the NADPH (circles) and NADH (triangles) concentration, both at aerobic (closed symbols) and anaerobic (open symbols) conditions.

As shown in Figure 2A, under aerobic conditions, the F_0/F_M ratio was barely affected by increasing the concentration of NAD(P)H. Under anaerobic conditions, the F_0/F_M ratio increased with increasing NAD(P)H concentrations, but the maximum extent of the increase was already achieved at 25 μM NADPH or 50 μM NADH. Concerning F_I/F_M, F_J/F_M, and $[d(F/F_M)/dt]_0$, it is clearly demonstrated that they increase with increasing NAD(P)H concentrations, however with significant differences between aerobic and anaerobic conditions (Figures 2B–D). The NAD(P)H induced increases under anaerobiosis appeared at much lower concentrations than under aerobic conditions and were saturated at about 25 μM and 1 mM, respectively. At saturating NAD(P)H concentrations, F_J/F_M and $[d(F/F_M)/dt]_0$ were significantly

higher under anaerobic conditions, but F_I/F_M was fairly comparable under both conditions. NADPH was more effective than NADH in altering the fluorescence transient under both atmospheric conditions, but only at sub-saturating concentrations. The incubation under anaerobic conditions without the addition of exogenous NAD(P)H to the chloroplast suspension resulted in an increase of F_0/F_M, F_I/F_M, F_J/F_M, and $[d(F/F_M)/dt]_0$ (Figure 2).

It is noteworthy that we purposely chose to deal only with the above phenomenological parameters, which simply quantify the differences between the fluorescence transients. We avoided to translate them to biophysical parameters, i.e. the maximum quantum yield of primary photochemistry $\varphi_{Po}=1-(F_0/F_M)$, the relative variable fluorescence $V=(F-F_0)/(F_M-F_0)$ at the different steps which is directly related to the fraction $B = Q_A^-/Q_{A,total}$ of closed RCs, and the initial slope $(dV/dt)_0$ which offers a measure of the corresponding initial rate $(dB/\Delta t)_0$ of the accumulation of closed RCs. The reason was that the F_0 level measured following a treatment with NAD(P)H does not represent the true F_0, which is defined as the fluorescence signal when Q_A is fully oxidised, i.e. when all RCs are in an open state. Further investigations are needed to clarify the origin(s) of the F_0 increase and, concomitantly, to determine the true F_0 which is necessary for the calculation of the biophysical parameters mentioned.

Summarising our findings, we can conclude that the redox state of the PQ pool, revealed by different fluorescence parameters, is determined by the amount of reducing equivalents (NADH or NADPH) and the presence or absence of oxidising equivalents (O_2). Even if a partial anaerobiosis is induced after the 1-h incubation with NAD(P)H, this cannot be, by itself, the reason for the wide changes of the fluorescence kinetics, since the complete (intentional) anaerobiosis induces changes that are much less pronounced. It is obviously expected that after 1-h incubation a portion of NAD(P)H is consumed to reduce PQ which is reoxidised by O_2 thus consuming a portion of it (possibly replaced by diffusion from the aerial phase in the vial). However, what is important for this study is not the actual amount of the reagents but the established equilibrium of the PQ pool redox state reflected on the fluorescence kinetics. In a future study continuing our investigations on the subject, we plan to incorporate measurements of the actual concentrations of NAD(P)H and O_2 after different incubation times, for each of the treatments investigated in our present study.

The NAD(P)H-dependent changes of the Chl a *fluorescence rise are insensitive to antimycin A and rotenone*

The experiments above presented show that both forms of reduced pyridine nucleotides are efficient in reducing PQ in the dark. Two hypotheses exist for the phenomena observed (see 'Introduction'): (1) The NADPH-induced reduction of PQ corresponds to the NADPH-specific PQ reduction observed in spinach chloroplasts and proposed to be realised via an electron transport pathway involving FNR and FQR (see e.g. Mills et al. 1979), while the PQ reduction by NADH follows another pathway, mediated by a chloroplastic Ndh complex. (2) Both NADPH- and NADH-induced PQ reduction are mediated by chloroplastic Ndh complex(es).

In order to check the first hypothesis, we used antimycin A which has been reported as inhibiting FQR (Mills et al. 1979; Endo et al. 1997,1998). We found that the NADPH-dependent changes of the Chl *a* fluorescence rise were insensitive to antimycin A (10 μM) both under aerobic and anaerobic conditions (data not shown). This indicates that, in our experiments, the NADPH-dependent dark reduction of PQ was realised via a pathway not involving FQR.

The second hypothesis was checked by using rotenone, a well-known inhibitor of the mitochondrial NADH-ubiquinone oxidoreductase (Trumpower 1981), which has also been reported to inhibit the activity of a photoactivatable NAD(P)H-dehydrogenase in isolated barley (*Hordeum vulgare*) thylakoid membranes (Teicher and Scheller 1998). However, we found that rotenone (1 mM) was inefficient in suppressing the NAD(P)H-induced changes of the Chl *a* fluorescence rise kinetics both under aerobic and anaerobic conditions (data not shown). It therefore appears that, in the dark, the non-photochemical reduction of PQ by NAD(P)H is mediated by an enzyme that is not sensitive to rotenone.

It can be argued that, since the above presented results for rotenone and antimycin A effect were obtained from measurements performed at the end of a 60-min incubation time, they do not exclude the possibility that the inhibitors might have affected the reduction rate of the PQ pool. However, as mentioned in the 'Materials and methods' section, similar results were obtained from measurements after a much shorter in-

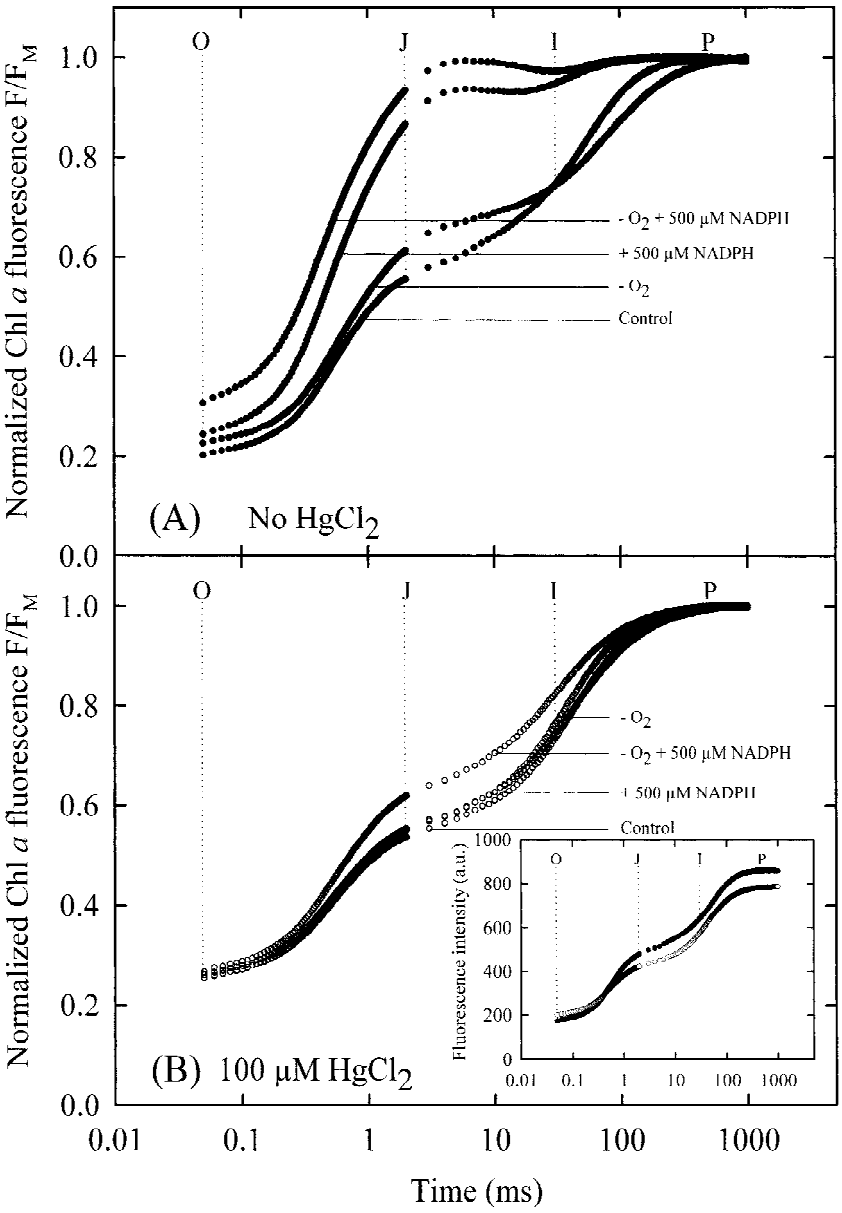

Figure 3. Chl *a* fluorescence rise kinetics of osmotically broken spinach chloroplasts that were incubated for 60 min at room temperature in the dark either under aerobic or anaerobic (– O_2) conditions, with or without a treatment with 500 μM NADPH and, with (B) or without (A) the presence of 100 μM $HgCl_2$ in the suspension medium. The transients were normalised on the corresponding F_M. The insert provides a comparison of the two control transients, i.e. the transients under aerobic conditions and without NADPH addition, with (○) and without (●) the presence of 100 μM $HgCl_2$. The transients in the insert are presented without any normalisation.

cubation time (15 min). Moreover, it can be reasonably stated that, if the inhibitors would have affected the rate of PQ reduction, this should have been revealed under aerobic conditions where a dynamic equilibrium is established between reduction of PQ by NAD(P)H and reoxidation of PQH_2 by oxygen; the redox state level of the PQ pool and, concomitantly, the fluorescence parameters tested, would not have been the same with and without the presence of the inhibitors. In our future investigation extending the present study where, as mentioned before, kinetic experiments for the determination of the rate of NAD(P)H-induced PQ reduction will be performed, the possible influence of

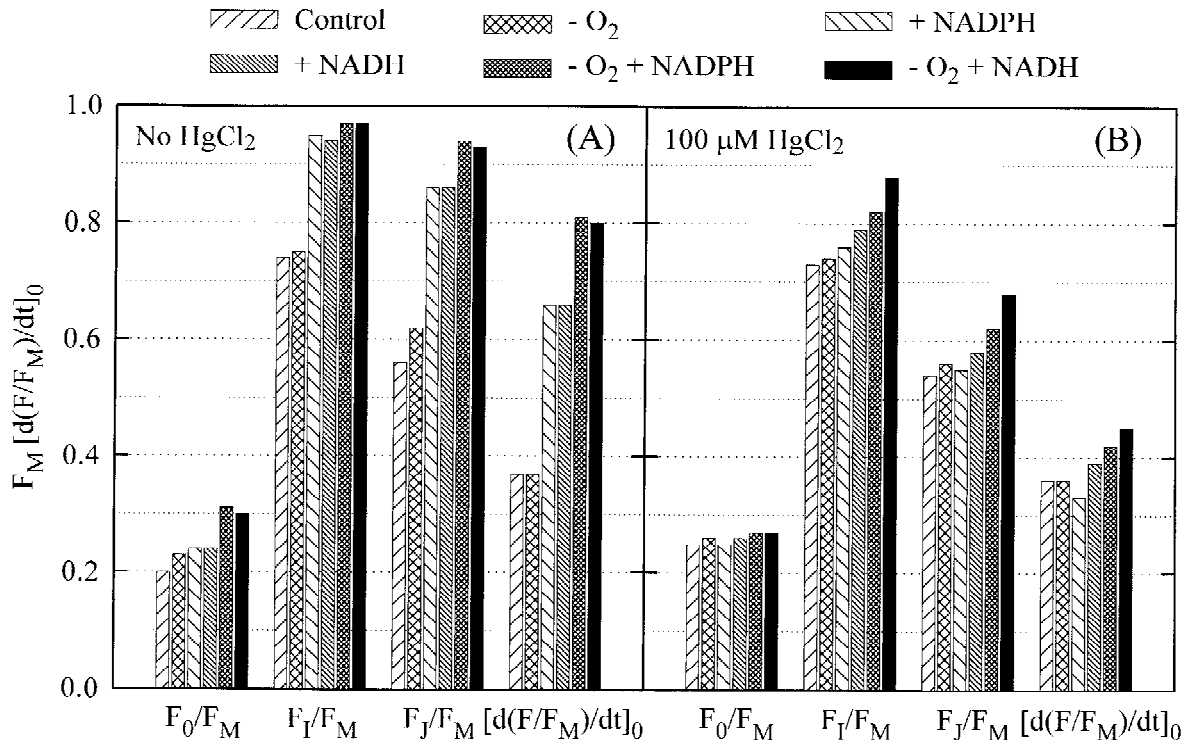

Figure 4. Selected parameters (see legend of Figure 2) characterising the fluorescence rise kinetics, calculated from the fluorescence transients obtained from osmotically broken spinach chloroplasts that were incubated for 60 min at room temperature in the dark either under aerobic or anaerobic ($-O_2$) conditions, with or without a treatment with 500 μM NADPH or NADH and, with (B) or without (A) the presence of 100 μM $HgCl_2$ in the suspension medium.

various inhibitors (including rotenone and antimycin A) on the rate will also be examined.

Inhibition by mercury of the NAD(P)H-dependent changes of the polyphasic Chl a *fluorescence rise kinetics*

The mercury ion Hg^{2+}, an inhibitor of the mitochondrial Ndh, has been shown to block also the electron transfer from cytosolic donors to the photosynthetic intersystem chain in the cyanobacteria *Synechococcus* and *Synechocystis*, while no such effect was observed in Ndh-defective mutants (Mi et al. 1992a,b; Tanaka et al. 1997). It was, therefore, of interest to check whether Hg^{2+} is competent in suppressing the NAD(P)H-induced non-photochemical reduction of PQ in higher plant chloroplasts.

Figure 3 clearly demonstrates that the addition of 100 μM $HgCl_2$ (corresponding to 0.55 mg $HgCl_2$/mg Chl) to the chloroplast suspension efficiently inhibited the changes of the shape of the Chl *a* fluorescence transient induced by the treatment of the thylakoids with 500 μM NADPH. Full inhibition was observed under aerobic conditions. Under anaerobic conditions, mercury abolished the NADPH-dependent increase in the O-level and severely inhibited, but not entirely eliminated, all the other NADPH-induced changes. The insert in Figure 3B shows that mercury did not basically change the shape of the fluorescence rise transient in the control sample, incubated under aerobic conditions in the normal suspension medium, though it slightly suppressed the amplitude of the variable part of the transient, due to a decrease of F_M and a minor increase of F_0. In order to present clearly this observation we chose to present in the insert of Figure 3 the actually recorded transients without any normalisation.

The utilisation of the parameters F_0/F_M, F_I/F_M, F_J/F_M, and $[d(F/F_M)/dt]_0$ characterising the Chl *a* fluorescence rise facilitates the comparison of samples that were incubated under aerobic or anaerobic conditions, with or without 500 μM NADPH or NADH, and with (Figure 4B) or without (Figure 4A) the presence of 100 μM $HgCl_2$ in the chloroplast suspension.

Concerning the F_0/F_M ratio, it is shown that, independently of the treatment, the addition of mercury

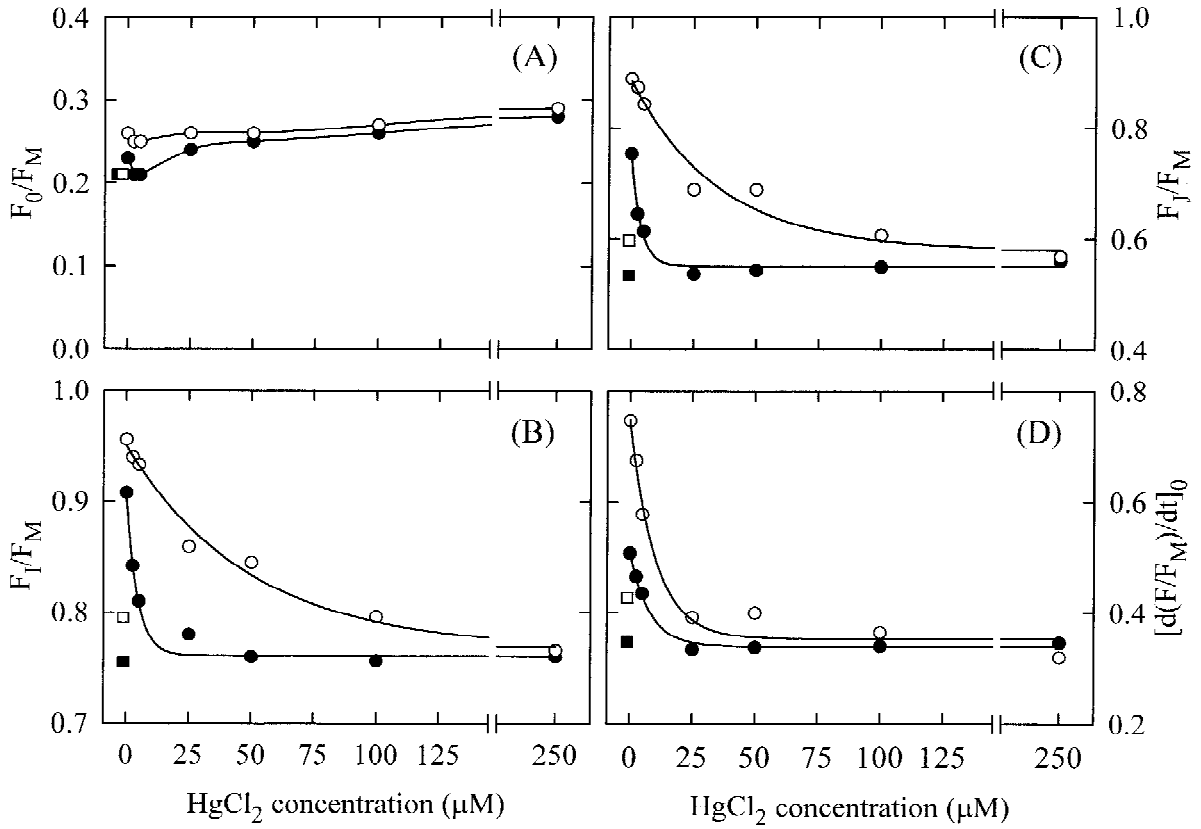

Figure 5. Effects of different concentrations of $HgCl_2$ on the selected parameters (see legend of Figure 2) characterising the fluorescence rise kinetics, calculated from the fluorescence transients obtained from osmotically broken spinach chloroplasts that were incubated for 60 min at room temperature in the dark in the presence of 500 μM NADPH. The experiment was conducted both under aerobic (●) and anaerobic (○) conditions. The detached symbols (■ for aerobic and □ for anaerobic conditions) correspond to samples where neither NADPH nor $HgCl_2$ were added (control samples).

results in almost the same values; their variation is restricted in the range from 0.25 (obtained for the control) to 0.28 (obtained in the presence of NAD(P)H under anaerobic conditions). Taking into account that these values are higher than 0.2 which is exhibited by the control without mercury, it can be concluded that we are witnessing two independent effects of mercury; the main effect is the suppression or even elimination of the NAD(P)H-dependent increase of the F_0/F_M ratio, while an additional and independent of the treatment effect on the values of F_M and F_0 (see insert of Figure 3) leads to an increase of the F_0/F_M ratio.

Mercury almost completely (aerobic conditions) or severely (anaerobic conditions) inhibited the NADPH and NADH-induced increases of F_I/F_M, F_J/F_M, and $[d(F/F_M)/dt]_0$. Under both aerobic and anaerobic conditions, the changes of F_I/F_M, F_J/F_M, and $[d(F/F_M)/dt]_0$ induced by NADPH were somehow more severely inhibited by mercury than those induced by NADH.

Figure 5 demonstrates the effect of different concentrations of $HgCl_2$ on the parameters F_0/F_M, F_I/F_M, F_J/F_M, and $[d(F/F_M)/dt]_0$ in chloroplasts incubated under aerobic (closed circles) and anaerobic (open circles) conditions in the presence of 500 μM NADPH. Similar results were obtained with NADH (data not shown). The detached symbols (closed squares for aerobic and open squares for anaerobic conditions) correspond to samples where neither NADPH nor $HgCl_2$ were added (control samples). Low $HgCl_2$ concentrations (about 5 μM) resulted in a full (aerobic conditions) or partial (anaerobic conditions) inhibition of the NADPH-induced increase of the F_0/F_M ratio; at concentrations higher than 5 μM, the F_0/F_M ratio increased however again with increasing concentrations under both aerobic and anaerobic conditions (Figure 5A), indicating that at higher con-

centrations mercury might have additional effects. An $HgCl_2$ concentration of about 25 μM was high enough to eliminate the NADPH-induced increases of F_I/F_M, F_J/F_M, and $[d(F/F_M)/dt]_0$ under aerobic conditions, as well as that of $[d(F/F_M)/dt]_0$ under anaerobic conditions (Figures 5B–D). However, under anaerobic conditions higher concentrations of $HgCl_2$ were required to eliminate the NADPH-induced increases of F_I/F_M and F_J/F_M.

Discussion

Measuring the kinetics of the Chl *a* fluorescence rise of osmotically broken spinach chloroplasts revealed that the addition of exogenous NADPH or NADH to the chloroplast suspension results in the accumulation of reduced PQ in the dark (Figure 1A). The finding that both forms of reduced pyridine nucleotide have the capability to reduce the PQ pool in the dark differs from the results obtained in an earlier study where NADPH-specific PQ reduction was observed in broken spinach chloroplasts (Endo et al. 1997). Furthermore, in other experiments using spinach (Mills et al. 1979) or tobacco (*Nicotiana tabacum*) chloroplasts (Endo et al. 1998), significant NADPH-induced non-photochemical reduction of PQ appeared only when exogenous ferredoxin was added to the chloroplast suspension, whereas the addition of ferredoxin was dispensable in the present work and other studies (Endo et al. 1997; Corneille et al. 1998). The NADPH-specific non-photochemical reduction of PQ is characterised by a sensitivity to antimycin A and was proposed as following an electron transport pathway involving FNR and FQR (Mills et al. 1979). In the present work we observed that the NADPH-induced reduction of PQ in the dark is not sensitive to antimycin A (data not shown). This indicates that, in our experiments, the NADPH-dependent dark reduction of PQ was realised via a pathway not involving FQR. Similar results were obtained in experiments performed with broken potato (*Solanum tuberosum)* chloroplasts (Corneille et al. 1998). Therefore, it appears that depending on the experimental conditions, the non-photochemical reduction of the PQ pool is mediated by different enzyme systems.

The finding that $HgCl_2$ efficiently inhibits the NAD(P)H-induced changes of the Chl *a* fluorescence rise transient (Figures 3 and 4) clearly demonstrates that mercury inhibits electron donation from NADPH and NADH to the PQ pool in broken spinach chloroplasts. This result shows an analogy with the blockage of the electron transfer from cytosolic donors to the photosynthetic intersystem chain in cyanobacteria (Mi et al. 1992a, b; Tanaka et al. 1997). However, to our knowledge, this is the first time that the dark reduction of PQ in higher plant cells is found to be suppressed by mercury.

On the other hand, incubation in the dark of isolated spinach thylakoid membranes in the presence of $HgCl_2$, in the same proportion to Chl (about 0.5 mg $HgCl_2$/mg Chl) as used in our study (Figures 3 and 4), was reported to result in a large reduction of the maximum quantum yield of the primary photochemistry of PS II as judged from a decrease in the F_V/F_M ratio; this decrease was the result of a strong suppression of the maximum fluorescence yield F_M, attributed to the inhibition by mercury of the oxygen-evolving complex at the PS II donor side (Boucher and Carpentier 1999). Contrary to this finding, we observed only minor effects of $HgCl_2$ on the F_V/F_M ratio: F_0/F_M under aerobic conditions and in the absence of NAD(P)H increased from to 0.2 to 0.25 by the addition of 100 μM $HgCl_2$, which means that the F_V/F_M ratio decreased from 0.8 to 0.75. Actually, as shown in the insert of Figure 3B, 100 μM $HgCl_2$ barely altered the shape of the Chl *a* fluorescence rise transient in our control samples, showing that mercury did not affect the photochemical activity of PS II. Mercury inhibition on the donor side of PS II has been shown to be partially reversed by chloride (Bernier et al. 1993). It was proposed that mercury exerts its action at a site related to chloride binding and/or function (Bernier et al. 1993; Bernier and Carpentier 1995). Hence, it is likely that in our experiments the high concentration of Cl^- in the suspension medium (42 mM Cl^- compared to 100 μM Hg^{2+}) protected the donor side of PS II against the inhibitory action of Hg^{2+}.

Our finding that Hg^{2+}did not modify the shape of the fluorescence transient indicates that mercury did not affect the electron flow through the intersystem electron transport chain and PS I; if Hg^{2+}, known to modify SH-groups and affect the photosynthetic apparatus at multiple sites [see Murthy et al. (1990) and references therein], had blocked this flow, the transients would have a shape reflecting a faster net reduction of Q_A due to the slowing down of Q_A^- reoxidation. Possible additional effects of mercury on an FNR-dependent pathway of non-photochemical reduction of PQ cannot be excluded; however, as above discussed, this pathway appears not to be involved in

the non-photochemical reduction of PQ we observed under our experimental conditions.

It can be therefore stated that, though mercury is known to be quite unspecific, our experiments revealed specifically one major effect, namely the inhibition of an FNR independent PQ reduction by NAD(P)H, indicating that all other possible influences of mercury on the photosynthetic apparatus were either minor (slight change in the F_0/F_M ratio) or did not interfere in phenomena revealed during the 1 s fluorescence transient.

The finding that Hg^{2+}, an inhibitor of the mitochondrial NADH-ubiquinone oxidoreductase, efficiently suppressed the non-photochemical reduction of PQ by NADPH and NADH, supports the idea that the dark reduction of PQ in higher plant chloroplast is mediated by a complex I-like enzyme. The Ndh complex recently purified from pea thylakoid membranes has been demonstrated to be an NADH-specific dehydrogenase (Sazanov et al. 1998b). This implies that if this is the enzyme mediating the NAD(P)H-dependent dark reduction of the PQ pool, then there must be a transhydrogenase or an indirect substrate cycle to explain the NADPH-dependent reduction of PQ that is not sensitive to antimycin A (Burrows et al. 1998; Endo et al. 1998). Alternatively, higher plant chloroplasts may contain an additional Ndh complex that specifically oxidises NADPH. Such an NADPH-specific dehydrogenase has been purified from *Synechocystis* (Mi et al. 1995; Matsuo et al. 1998). If higher plant chloroplasts contain two types of Ndh complexes, their relative abundance may differ among species and/or depend on growth conditions. This would give an explanation why in our experiments NADPH was somewhat more efficient than NADH in reducing PQ (Figures 1 and 2), whereas in experiments with potato chloroplasts NADH was significantly more efficient than NADPH (Corneille et al. 1998).

On the other hand, the finding that the dark reduction of PQ by NADPH and NADH was not sensitive to rotenone (data not shown) would argue against the participation of a complex I-type enzyme. However, the rotenone sensitivity of purified chloroplast Ndh complex has not yet been investigated. A further possibility (see Corneille et al. 1998) would be that the non-photochemical reduction of PQ by NAD(P)H is mediated by a DT-diaphorase-like enzyme; this enzyme, a flavoprotein, is a rotenone-insensitive NADH-quinone oxidoreductase which catalyses an obligatory two electron-reduction of quinone using either NADPH or NADH.

Rotenone has been shown to strongly suppress the photoactivated post-illumination non-photochemical reduction of PQ in sunflower leaves (Feild et al. 1998) and to inhibit the activity of a photoactivatable NAD(P)H dehydrogenase in isolated barley thylakoid membranes (Teicher and Scheller 1998). Therefore, it is possible that the light activated non-photochemical reduction of PQ is mediated by a complex I-type enzyme, whereas the dark reduction of PQ which does not require light activation is mediated by a DT-diaphorase-type enzyme that is sensitive to mercury. The existence of a rotenone-insensitive pathway for dark PQ reduction would be consistent with the finding that net dark reduction of PQ was still possible after rotenone treatment in sunflower leaves when dark reoxidation of reduced PQ was inhibited by fumigation with CO (Feild et al. 1998).

The finding that NADPH and NADH-dependent dark reduction of PQ is strongly enhanced by anaerobiosis (Figure 1B) supports the idea of the occurrence of chlororespiration in the thylakoid membranes of higher plant chloroplasts. The NAD(P)H-dependent changes of the kinetics of the Chl *a* fluorescence rise saturated at about 25 μM and 1 mM under anaerobic and aerobic conditions, respectively (Figure 2). This result, along with the observation that at saturating NAD(P)H concentrations the PQ pool was in a higher state of reduction under anaerobic than under aerobic conditions (Figures 1 and 2), provide additional evidence that the putative chlororespiratory electron transport chain comprises a terminal oxidase which, in the presence of O_2, reoxidises reduced PQ in the dark. In this respect, a study with *Chlamydomonas reinhardtii* has presented new evidence for the presence in the thylakoid membranes of an oxidase that catalyses the oxidation of plastoquinol and the reduction of oxygen to water (Cournac et al. 2000). The finding that higher concentrations of $HgCl_2$ were required under anaerobic conditions than under aerobic to suppress NADPH-dependent dark reduction of PQ (Figure 5) supports the hypothesis that the redox state of the PQ pool in the dark is regulated by the rates of non-photochemical PQ reduction and O_2-dependent oxidation of reduced PQ. Thus, the recent finding that in broken pea chloroplasts NAD(P)H-induced dark reduction of PQ appeared only under anaerobic conditions (Haldimann and Strasser 1999) could be attributed either to a less efficient PQ reduction or to a more efficient reoxidation of reduced PQ. In freshly broken chloroplasts donation of electrons from NADPH to PQ is characterised by a very labile activity (Endo et al. 1997). Thus, it is possible that pea,

as compared to spinach, was more sensitive to inactivation of the NAD(P)H-PQ oxidoreductase during the preparation of the thylakoids. Differential sensitivity to inactivation could also be the origin of the differences we observed under aerobic conditions among different preparations with respect to the extent of the NAD(P)H-induced PQ reduction (compare Figures 1A and 3A). However, it is equally likely that such differences among plant species, or within the same species grown under different conditions, originate from differences in the abundance and/or the activity of the enzymes (i.e. the NAD(P)H-PQ oxidoreductase at the PQ reducing side and the putative oxidase at the PQ oxidising side) determining the reduction state of the PQ pool in the dark.

In many studies dealing with the phenomenon of chlororespiration, the non-photochemical reduction of PQ was monitored by measuring an increase in the apparent F_0 under weak non-actinic light that results from the accumulation of Q_A^- as consequence of the accumulation of reduced PQ in the dark. However, according to the theoretically derived and experimentally verified dependence of $Q_A^-/Q_{A,tot}$ on PQH_2/PQ_{tot} (Diner 1977), if a small fraction of PQ is reduced, the reduction of Q_A is so limited that the resulting increase of the apparent F_0 may be undetectable. The utilisation of the Chl *a* fluorescence rise kinetics offers a good alternative to overcome such limitations and detect dark PQ reduction, even of small extent.

Concluding remarks

In conclusion, we have shown that in broken spinach chloroplasts both forms of reduced pyridine nucleotides, NADPH and NADH, have the capability to reduce PQ in the dark and that this reaction is suppressed by mercury. To our knowledge, this is the first demonstration that the non-photochemical reduction of PQ can be efficiently suppressed by mercury in higher plant chloroplasts. Furthermore, the result that the NAD(P)H-dependent dark reduction of PQ is strongly enhanced by anaerobiosis supports the view of the existence of a PQ-oxidase in the thylakoid membranes. Overall, our data are consistent with the presence of a chlororespiratory electron transport chain in higher plant chloroplasts and demonstrate once again that measuring the kinetics of the Chl *a* fluorescence rise is a valuable tool to monitor dark reduction of the PQ pool.

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